Original Article

ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 3 – Data Analysis, Reporting and Case Studies

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Over the past several years, a diverse group of physicians and other laboratory scientists have developed various recommendations and guidelines regarding best practices for PNH testing. This manuscript is based on these previous recommendations as well as various other relevant publications of experts in the area of PNH testing. The goal is to provide flow cytometry laboratories with an updated consensus approach to analysis and reporting of PNH results and to address the most common analytical challenges for accurate reporting of this rare disease. A comprehensive case library is included in this section. **© 2017 International Clinical Cytometry Society**

Key terms: PNH; paroxysmal; nocturnal; hemoglobinuria; flow cytometry; monocytes; neutrophils

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INTRODUCTION

A correct diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) is essential for effective patient management and possible treatment if clinically indicated (1–3). Although flow cytometry has become the gold standard for the diagnosis and monitoring of patients with PNH and related diseases (4–7), it is important to emphasize that flow cytometry by itself does not establish the clinical diagnosis of overt/clinical PNH as this is determined by a number of other clinical factors in addition to the finding and quantification of glycosylphosphatidylinositol (GPI)-deficient cells by flow cytometry (8,9). High sensitivity flow cytometric analysis can identify the presence of PNH phenotypes and is able to quantify the PNH clone size in red blood cells (RBCs) and white blood cells (WBCs, neutrophils, and monocytes) down to a lower limit of quantification (LLOQ) of 0.01% for RBC and 0.05–0.1% for neutrophils. The PNH clone size in WBCs is typically used to determine the extent of disease. Historically, the neutrophil clone size was used for this purpose based on the absence of at least two GPI-linked structures from neutrophils. However, more recently many laboratories have noted the additional value of assessing and reporting the PNH clone size in the monocytes as well. Note that we use the more appropriate term "neutrophils" instead of the historical term "granulocytes" throughout this document. Modern

Additional supporting information may be found in the online version of this article.

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gating techniques based on light scatter, CD45 and CD15 (Part 2, Sutherland DR et al) result in exclusion of basophils and most eosinophils (although not bands) from the population of interest so that for the most part "granulocyte" clones are in fact "neutrophil" clones. Nevertheless, some figures may still be labeled as granulocytes or "grans."

The need for high-sensitivity flow cytometric testing is demonstrated through a study of a large patient population over a 9-year period, which investigated the distribution of PNH clones from minor clones (<1% in the WBC) to the larger PNH clones sizes (Fig. 1). These findings were based on 10,236 patient samples (excluding follow-up samples) over a 9-year period at a single laboratory, which showed the incidence of PNH clones to be 6.2% (633 patients) of the total screened patients among high risk patient groups (8,9,25). This prevalence value has consistently been in the 6–7% range and is in agreement with other studies which have found a similar PNH clone prevalence (unpublished data by Luigi DelVecchio). As shown in Figure 1, 363 out of 10,236 total patients (3.6%) showed a PNH clones >1% and 270 out of 10,236 total patients (2.6%) showed a minor PNH clone of <1%. Thus 42% of patients with a PNH clone show a clone of <1%. PNH clones are referred to as minor (14,16) when the clone size is <1% of any lineage (case library, Case 3) and such are often seen in patients with Aplastic Anemia (AA) and some subsets of myelodysplastic syndromes (MDS). Although patients with these minor PNH clones most likely do not exhibit clinical symptoms of PNH, the detection of smaller PNH clones in patients with subclinical PNH associated with bone marrow failure syndromes (8–11) requires subsequent monitoring at defined intervals to monitor potential clone expansion and possible transition to classical hemolytic PNH (12). The presence of minor PNH clones in patients with AA is associated with better response to immunosuppressive therapy (24).

This high percentage of patients with PNH clones of <1% emphasizes the need for high-sensitivity PNH testing in all three lineages: RBC, neutrophils, and monocytes.

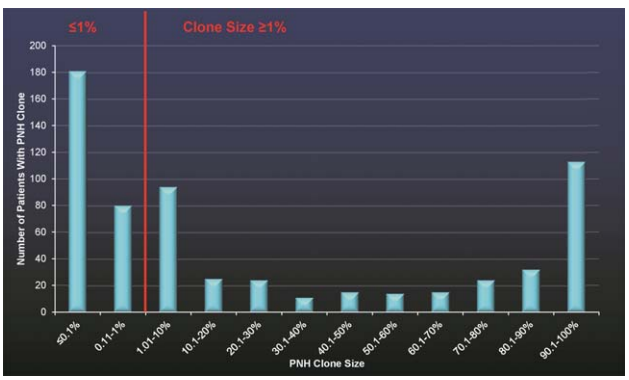


FIG. 1. PNH neutrophil clone size distribution (633 PNH+ patients/total of 10,236 screened patients based on the diagnostic pathway) showing 42% of PNH+ patients with a PNH clone of <1%. Data from Dahl-Chase Diagnostic Services (December 2008–October 2016). [Color figure can be viewed at wileyonlinelibrary.com]

The PNH clone size in RBCs is typically lower in untreated patients with PNH due to hemolysis and/or transfusion. The difference between the PNH clones sizes in the WBCs and RBCs is often related to the extent of hemolysis as the GPI-deficient RBCs are destroyed by complement-mediated lysis. Although several attempts have been made to determine a "threshold PNH clone size" for a diagnosis of clinical PNH (10–20%), it should be noted that patients with smaller clone sizes in the WBCs can show signs of clinical PNH while some patients with larger clone sizes may not show the expected clinical signs of the disease as typically observed in patients with classical PNH. Accurate assessment of PNH clone in all three lineages (RBC, neutrophils, and monocytes) is becoming the standard of care for patients with suspected PNH. Modern flow cytometers have at least five photomultiplier tube detectors (PMTs) and a single tube 5C or 6C WBC assay can accurately and cost-effectively assess the presence of a PNH clone in both WBC lineages (neutrophils and monocytes) and a single two-color tube can assess the RBC lineage. This is a rare disease and many variations exist, which make it risky to assess only one lineage which may not be representative of the true PNH clone size. Evaluation of all lineages allow for complete assessment, especially when interpretation of the results in some lineages may be technically challenging, thus ultimately resulting in better patient management.

DISTINGUISHING PNH POPULATIONS FROM NORMAL CELLS

Because PNH clones frequently constitute only a very small fraction of total red blood cells or white blood cells there are special consideration that govern analysis and interpretation of data. There are no absolute "rules" for how many cells need to be assayed or how many PNH events must be counted in order to report the presence of a PNH population. However, several factors must be taken into consideration:

1. "Background rate," or limit of blank (LOB), i.e., the percentage of phenotypic PNH cells detectable in normal individuals. In general, this has been determined to be at least 5–10 fold lower (and even more for RBCs) than the 0.01% value targeted for reporting although it must be considered in the assessment of sensitivity. The incidence of phenotypic PNH cells is approximately 2–6/million acquired gated events in the RBCs. The incidence of phenotypic PNH cells in neutrophils is 2–10/million (Table 5 in Part 4).
2. Assay-related artifacts. While the use of multiparameter gating in the assays described here usually generate very "clean" data, each laboratory should assay normal samples to ensure that their background rate is significantly lower than their cutoff level (LOD or LLOQ) for a reportable population of PNH cells. It may be difficult in some circumstances to distinguish whether a small number of events represent a "real" population or an artifact. The best way to make this

Table 1
Estimated LLOQ and LOD According to the Total Number of Cells Acquired

Total number of gated cells acquired	Quantitative assay using LLOQ (>50 PNH cells) (%)	Qualitative assay using LOD (>20 PNH cells) (%)
10,000	0.5	0.2
20,000	0.25	0.1
30,000	0.17	0.066
40,000	0.125	0.05
50,000	0.1	0.04
100,000	0.05	0.02
200,000	0.025	0.01
300,000	0.017	0.007
400,000	0.0125	0.005
500,000	0.01	0.004
1,000,000	0.005	0.002

distinction is often to collect more events; if doubling the number of cells collected does not result a commensurate increase in the number of PNH events, or if the distribution of the new events is different from that seen originally, one should be very cautious when interpreting the results.

3. Statistical considerations. These are primarily of two types.
   a. The first relates to the statistics of small numbers as governed by the Poisson distribution, and can be used to determine the probability that finding a certain number of events is greater than would be expected in a normal individual (i.e., that a PNH population is present), without determining the actual percentage of events there are. If the background rate is 1/100,000 cells, finding five "real" events out of 100,000 cells (with the caveats about real events noted in #2 above) has a >99% probability of being a true positive. However, in order to mitigate concerns about artifacts, we recommend that 20 definitive GPI-negative events be considered the minimum number of events required to report a detectable population of PNH cells (provided, of course, that the percentage of GPI-negative events remains significantly greater than the background rate). This minimum percentage detectable above background is the lower limit of detection (LOD) and can be calculated as 20 PNH cells × 100/number of gated events acquired (Table 1). Even though statistical arguments indicate a much lower theoretical LOD, to date there are no clinical data to support the need to achieve these. However, there may be value, particularly in the context of clinical trials, for those laboratories with clean assays who can demonstrate reproducibility of a lower LOD to employ this so that the clinical utility of distinguishing very low levels of disease from absence of disease can be studied.
   b. The second refers to the precision with which the actual percentage of PNH cells can be determined. Again, Poisson statistics governs the relationship between the standard deviation of a distribution and the number of events counted (N) given by SD = 100/SQRT (N). Counting 16 events yields a standard deviation of 25%; 25 events yields a standard deviation of 20%; 50 events a standard deviation of about 15%; 100 events a standard deviation of 10%. There are not data in the literature to know what precision is required for clinical purposes at the low end of sensitivity although if these data are ever to be obtained the higher the assay precision the more likely that this can be investigated. We recommend collecting a minimum of 50 events for quantitation purposes in most circumstances, and especially for inexperienced laboratories, although as few as 25 events could be considered acceptable provided laboratories demonstrate that they in fact achieve the theoretical level of precision of 20%. The LLOQ is then calculated as 50 PNH cells × 100/number of gated events acquired and a quantifiable population of PNH cells can be reported.

RBC ASSAY

Acquisition and Analysis

For high-sensitivity RBC analysis, the ICCS Guidelines recommended the use of CD235a (for RBC gating) and CD59 (to detect GPI-deficient cells) (9). Based on subsequent publications that included rigorous testing and validation of various CD235a and CD59 clones and conjugates, optimal reagent combinations of CD235a-FITC and CD59-PE were identified (Part 2, Table 1, Sutherland DR et al). Once extensively titrated, these reagents in combination did not cause major aggregation of RBCs while still maintaining a good signal-to-noise ratio and the ability to adequately separate Type II and Type III PNH RBCs from normal (Type I) RBCs (13,14,16).

Red blood cells are analyzed by a series of gating dot plots beginning with TIME versus side scatter (SS) (Fig. 2a), forward (FS) versus side scatter (Fig. 2b) with detectors set in logarithmic mode and CD235a-FITC versus FS (Fig. 2c) to gate singlet RBCs and to quantify and exclude any remaining RBC aggregates. TIME is collected as a parameter (as is recommended for all clinical flow assays) and monitored during acquisition so that if fluidics problems are encountered, the sample can be re-acquired if possible, or if not, data acquired prior to the fluidics hiatus can be "gated" and only that portion

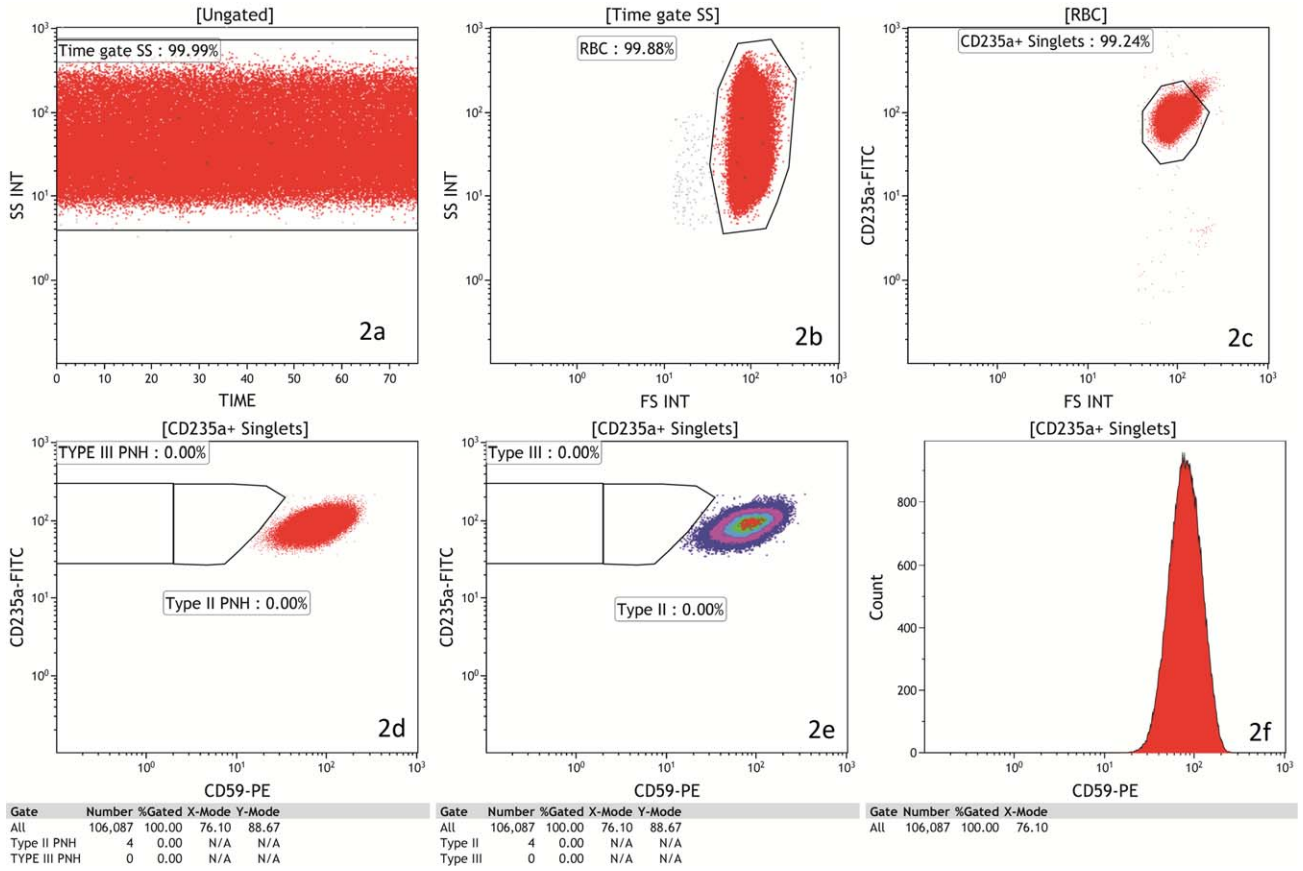


FIG. 2. Patient with no evidence of PNH clone. [Color figure can be viewed at wileyonlinelibrary.com]

of the data file subsequently analyzed. It is important to adjust the threshold (discriminator) for the forward scatter so that no RBCs are excluded from acquisition. The diagnostic plots include a bivariate CD59 versus CD235a dot plot (Fig. 2d), a bivariate CD59 versus CD235a density plot (Fig. 2e) and a single parameter histogram of CD59 staining (Fig. 2f). Bivariate dot plots and/or density plots are recommended over single-parameter histograms, especially for samples containing small numbers of PNH phenotypes, for identifying poorly stained samples that need to be re-stained, and for detecting media contamination and troubleshooting instrumentation issues (13 and Supplementary Data). However, while data regarding clone sizes comes predominantly from the two-dimensional plots, in which the gating regions are linked across the dot-plot and density plots, the single parameter histogram can also be useful in some situations. All three plots work in concert for optimal adjustment of the regions for Type III PNH cells and Type II PNH cells. An additional utility of the single parameter histogram is in comparing old versus new lots of CD235a-FITC/CD59-PE cocktails when tested on non-PNH samples.

Patients with PNH clones may show cells with partial CD59 deficiency (Type II) and/or complete CD59 deficiency (Type III). Figure 3 shows a clear delineation between Type II and Type III RBC clusters which allows for reporting of these subsets separately as well as quantifying the total PNH clone size (Type II and Type III combined). It should be noted that *bona fide* PNH RBCs express CD235a at levels comparable or even slightly higher than that expressed on normal Type I RBCs. This finding is quite useful as decreased CD235a and decreased CD59 expression usually indicates a staining artifact rather than true Type II RBCs (see troubleshooting section and Fig. 6 in Supplementary Data of 13).

Dynamic Region Setting for Type I, Type II PNH, and Type III PNH

When initially establishing the RBC assay, the Type III gate is positioned using cells stained only with CD235a-FITC, while the approximate location of the upper limit of the Type II region is determined by a normal sample stained with CD235a-FITC and CD59-PE (Fig. 1 of Part 2 Sutherland DR at el). In most cases the PNH clusters are evident and the initial region settings can be used for the quantification of the Type II and Type III RBC cells based on a combination of bivariate dot plots, density plots and the single parameter CD59 histogram (Fig. 4a). However, in some patients the regions may need to be adjusted (dynamic region setting) based on slight patient-specific differences in the CD59 expression rather than

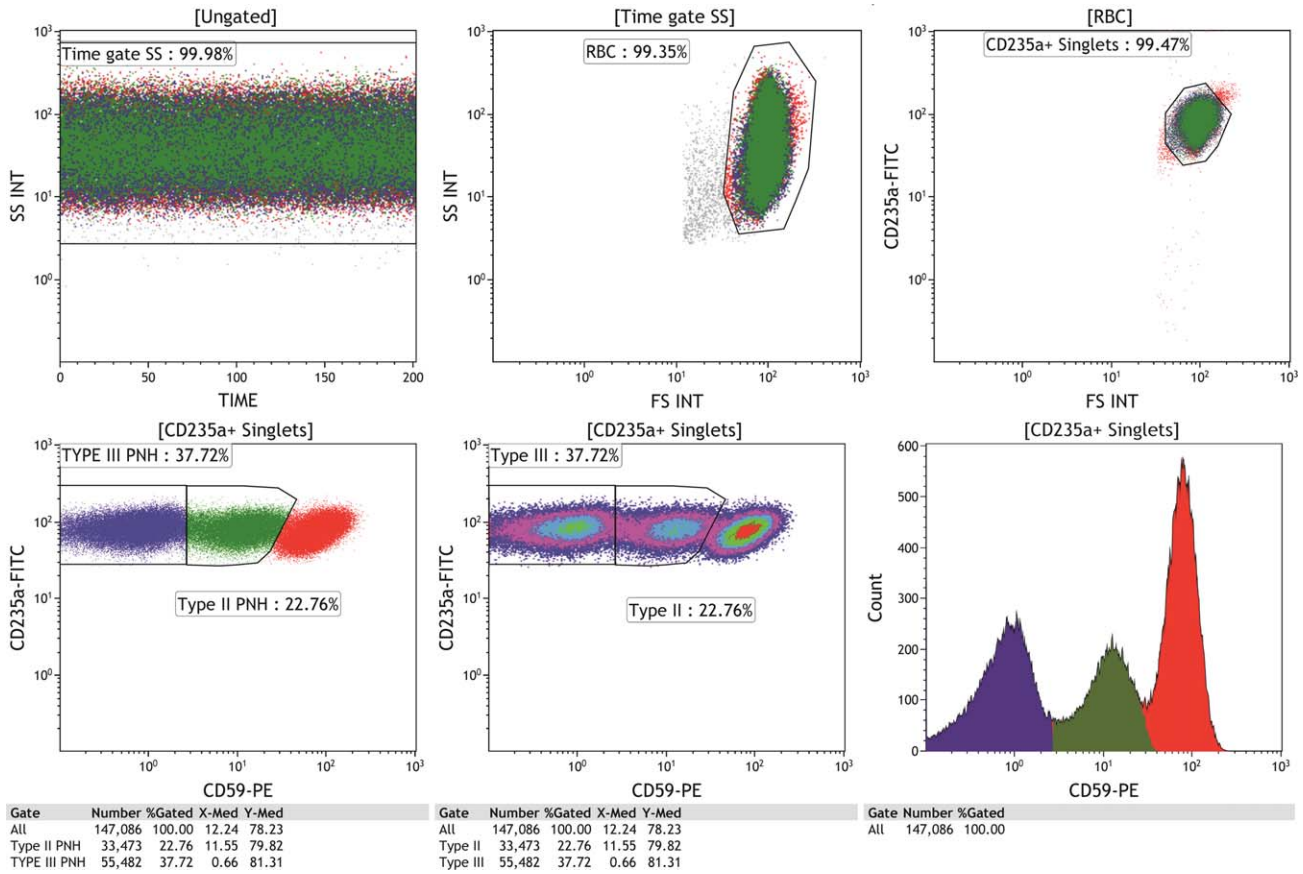


FIG. 3. Patient with PNH RBC clone comprised of distinct Type II and Type III PNH RBC clones.

strictly adhering to the initial region setting (Fig. 4b). Occasionally there is no clear distinction (clustering) between PNH Type II and PNH Type III (Fig. 4c) or between PNH Type II and normal Type I cells (Fig. 4d) which renders the interpretation challenging and the PNH clone size assessment and reporting potentially problematic. In such cases it may be appropriate to include a descriptive comment in the report and state that the percentages provided are an estimate only. In cases with unclear separation between Type III PNH cells and Type II PNH cells, it is recommended to report a combined population of RBCs with CD59 deficiency (Fig. 4c) with a comment describing the reason, rather than setting arbitrary regions for Type II and Type III. Patients with poor separation between PNH Type II and normal Type I RBCs are especially challenging as the region setting between these two populations will affect the total combined PNH clone size. Reasons for this poor separation may include the presence of microcytes (smaller RBCs) in certain patients (case library, case 13), transfusion (transfused cells typically have slightly decreased CD59 expression) and other unexplained decrease in CD59 expression (Fig. 4b). In these cases a static region setting (based on the initial region setting) would not work and a more flexible region setting based on the delineation of cell clusters is recommended (Fig. 4b). It should be noted that it is important to get a recent transfusion history if possible at all. This is particularly important for those patients where the Type II PNH cells are very bright and merge with normal CD59 expression. For these patients at presentation, an accurate assessment of the PNH clone is almost impossible without knowing if the patient has had any RBC transfusions. In cases of extreme iron deficiency or red cell fragmentation, with or without transfusions to complicate matters, the CD59 expression on the Type I population can be bimodal. Certain patients exhibit poor separation between the Type III PNH, Type II RBCs and the normal RBCs (Fig. 4d) and the region setting is somewhat challenging. It should be mentioned in the report that the percentages are an estimate only. In some cases there is no separation at all between the Type II PNH and normal RBCs and rather than setting arbitrary regions, it is recommended to not report a percentage for the PNH Type II cells (Fig. 4e) and include a comment describing the reason. In cases of transfused patients, it may be difficult to determine the true percentage of PNH Type II RBCs due to an overlap of transfused cells, which often show decreased CD59 staining. For these situations, the addition of CD71 may be helpful, as gating on the nucleated CD71-positive RBCs allows for gating out the donor's transfused cells. A detailed protocol is provided

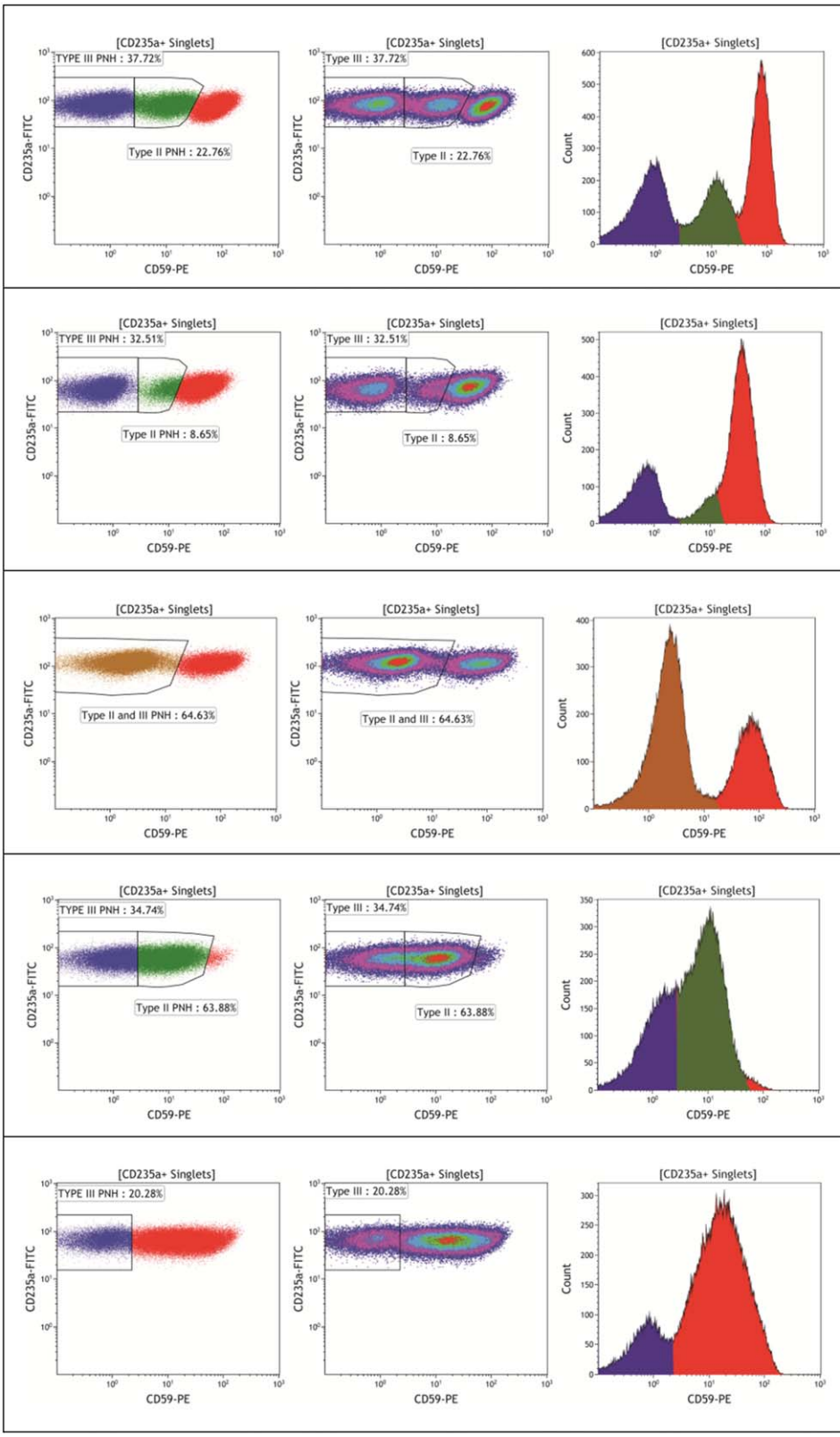

[CD235a+ Singlets]
TYPE III PNH : 37.72%
Type II PNH : 22.76%
Type III : 37.72%
Type II : 22.76%
CD235a-FITC
CD59-PE
Count
TYPE III PNH : 32.51%
Type II PNH : 8.65%
Type III : 32.51%
Type II : 8.65%
Type II and III PNH : 64.63%
Type II and III : 64.63%
TYPE III PNH : 34.74%
Type II PNH : 63.88%
Type III : 34.74%
Type II : 63.88%
TYPE III PNH : 20.28%
Type III : 20.28%

Figure 4a:

PNH Type II and Type III clone assessment based on initial region setting in the dotplot and density plot. The percent PNH clone is derived from the bivariate plots.

The CD59 single parameter histogram is shown for comparison, supporting this region setting.

Figure 4b:

PNH Type II and Type III clone assessment with slight patient-specific region adjustment for the PNH Type II region.

The CD59 single parameter histogram is shown for comparison, supporting this adjustment.

Figure 4c:

PNH Type II and Type III clone assessment as a combined PNH RBC clone due to unclear region setting for PNH Type III and PNH Type II cells.

The CD59 single parameter histogram is shown for comparison and illustrates the poor delineation between PNH Type II and PNH Type III.

Figure 4d:

This PNH+ patient shows no clear separation between PNH Type III, PNH Type II and normal Type I cells. Percentages are reported as an estimate only.

The CD59 single parameter histogram is shown for comparison and supports the delineation of PNH Type III, Type II and Type I to some extent.

Figure 4e:

This PNH+ patient shows no separation between PNH Type II and normal Type I cells. The PNH Type III population can be reported accurately (20.3%) but no percentage for the PNH Type II cells can be provided.

The CD59 single parameter histogram is shown for comparison and does not aid in the delineation of Type II/Type I.

FIG. 4. Various RBC plots with interpretational difficulties for the region settings between normal Type I RBC and PNH Type II and PNH Type III RBC populations.

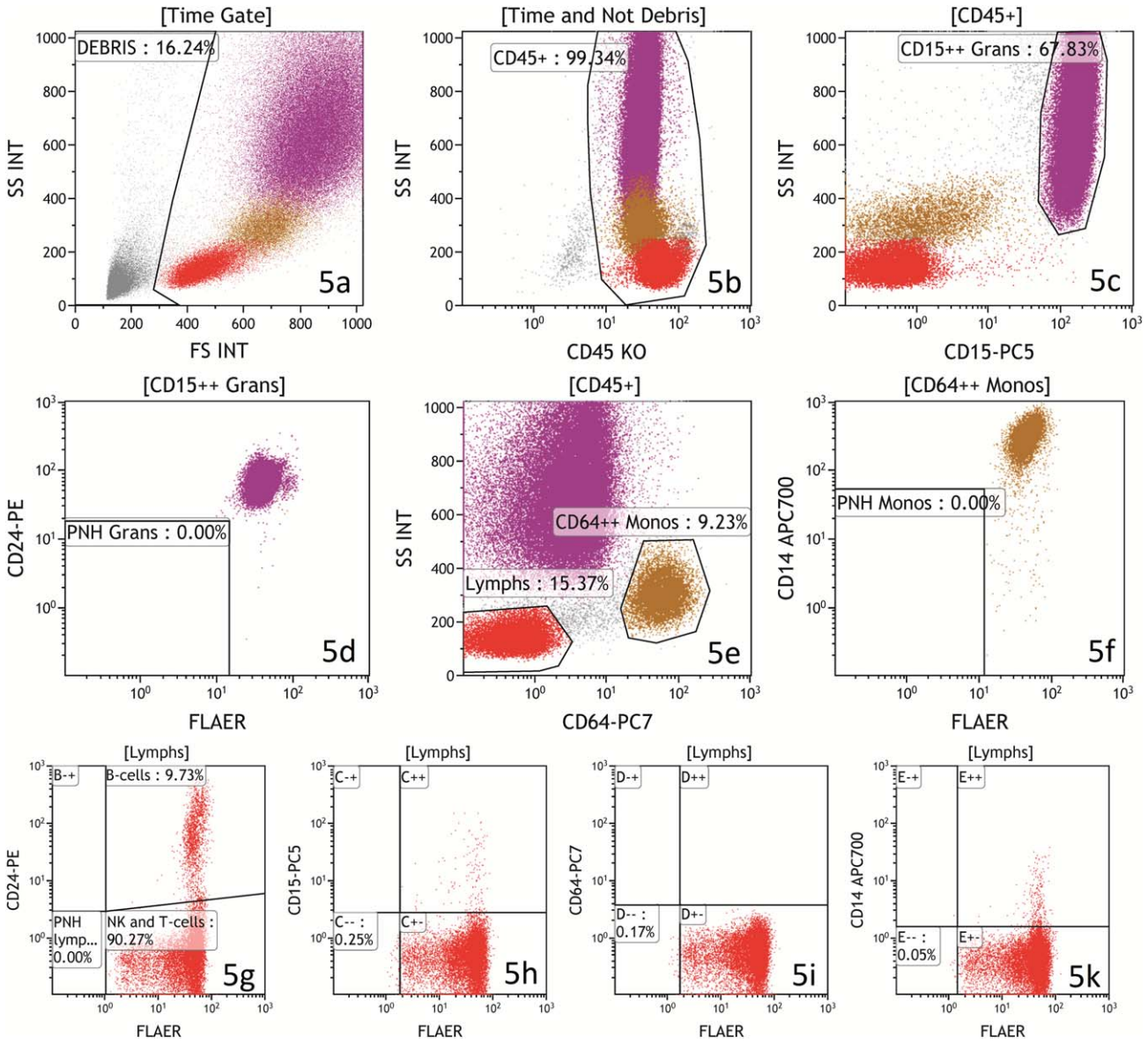


FIG. 5. Patient with no evidence of PNH cells in the neutrophils and the monocytes. **5a through 5f** illustrate the gating strategy and diagnostic plots for the determination of the PNH clone size in the neutrophils and the monocytes. **5g through 5k** describes the use of lymphocytes as internal control for verification of voltage settings, compensation and antibody performance. [Color figure can be viewed at wileyonlinelibrary.com]

in the *Current Protocols of Cytometry* (16) and illustration of relevant plots can be found in case study 16 of the case library. For reasons not fully understood, occasional non-PNH samples may also show a slight decrease in CD59 staining intensity and in this setting, flow cytometry cannot distinguish between *bona fide* PNH Type II cells and normal RBCs (see case library, case 13).

Recommendations for Data Acquisition

The following illustrates a practical approach for the detection of PNH RBCs down to a LLOQ of 0.01% based on both visual inspection of the plot as well as acquisition of an adequate number of events within this high-sensitivity PNH RBC assay when necessary. The acquisition of 100,000 RBCs is sufficient for most cases but more cells need to be acquired if necessary.

1. If greater than 1% PNH cells are identified within the Type II and Type III regions, the acquisition of 100,000 events should be sufficient to generate statistically reliable data and allow optimal region setting for Type III and/or Type II PNH populations.
2. If there are no cells with PNH phenotype detected in first 100,000 gated RBCs, the acquisition can be stopped and the RBCs can be reported as "no PNH cells detected." Based on Poisson statistics, there is a greater than 99.9% probability that the true frequency

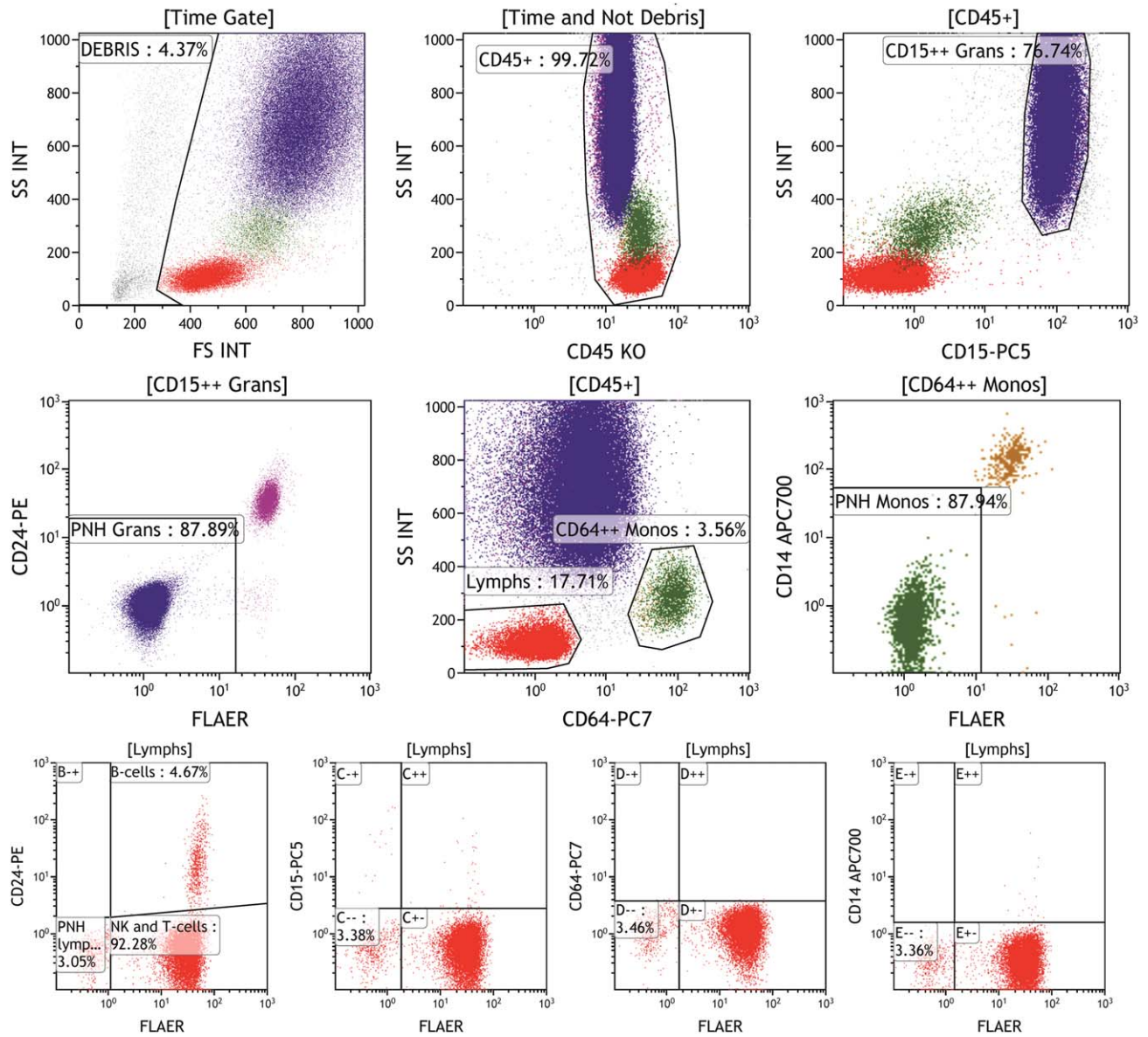


FIG. 6. Patient with a large PNH clone in the neutrophils and the monocytes. Top 2 rows describe the gating strategy and diagnostic plots for the determination of the PNH clone size in the neutrophil and the monocytes. Bottom row describe the use of lymphocytes as internal control for verification of voltage settings, compensation, and antibody performance. PNH lymphocytes are a visible cluster in the dual-negative region. [Color figure can be viewed at wileyonlinelibrary.com]

of GPI-deficient cells is less than 0.01% at this level of acquired cells.

3. If less than 1% *bona fide* PNH RBCs are identified with the CD59-negative/CD235a++ phenotype within the first 100,000 events, the acquisition should be extended if necessary such that a statistically reliable number of PNH events is obtained, as discussed above. These minor PNH populations are illustrated in Figure 13b,c. It is important to verify these events as *bona fide* PNH phenotypes versus a staining artifact. True Type II and Type III PNH RBCs show high CD235a expression, which should show at least the same CD235a brightness as the normal Type I RBCs. If there are events in the type II region with decreased CD235a and CD59 expression, these are most likely due to inadequate staining and they should not be included as PNH phenotypes (see trouble-shooting section in Supplementary Data of 13). Inspection of the dot plot as described above results in a sensitive and specific PNH RBC assay.

   a. Using LLOQ: If more than 50 PNH cells are identified, a quantitative result (percentage) can be reported (Fig. 13b).

b. Using LOD: If at least 20 PNH phenotypes but less than 50 PNH phenotypes (as required for the LLOQ) are seen, it should be reported as "rare cells with PNH phenotype detected below the level of quantification" (Fig. 13c).

WBC ASSAY

High-sensitivity methodologies to detect PNH phenotypes in neutrophils and monocytes have been published previously (13–18). These methods were initally based on two separate four-color neutrophil (FLAER, CD24, CD15, and CD45) and monocyte (FLAER, CD14, CD64, and CD45) tubes. In this earlier setting, samples were stained first with the RBC and neutrophil cocktails and if PNH phenotypes were detected, the "reflex" monocyte tube was thereafter set up (13,14). The current guidelines will refer to the same gating strategy used in earlier assays but discuss the more modern single tube assays on newer flow cytometers with 5, 6, or more PMTs that allow the simultaneous detection and quantification of both neutrophils and monocytes.

FLAER/CD24/CD14 Based Single Tube Assay for Neutrophils and Monocytes

For laboratories equipped with modern cytometers with 6, 8, or 10 PMTs (Canto, Canto II, and Navios), it is possible to configure six-color cocktails based on FLAER, CD24, and CD14 (Table 2 and 3 of Part 2, Sutherland DR et al). As for previously published 4-C assays, analysis begins with review of TIME versus CD45 or Side Scatter on ungated events (not shown) to confirm that no fluidics irregularities are present. Figure 5 shows the sequence of bivariate gating dotplots (5a, 5b, 5c, and 5e) from a Navios-specific reagent set comprising FLAER-Alexa488, CD24-PE (clone ALB9), CD15-PC5 (clone 80H5), CD64-PC7 (clone 22), CD14-APCA700 (clone RMO52), and CD45-KO (clone J33). Diagnostic dotplots for the detection and quantification of a PNH neutrophils and PNH monocytes are shown in plots 5d and 5f, respectively. As published previously (13–17), the FS versus SS plot is gated from TIME versus SS plot (not shown) and light scatter voltages are set so that all WBC subsets are clearly visible and optimally separated (Fig. 5a). The threshold/discriminator is set to ensure that no lymphocytes are excluded. A debris exclusion gate (or WBC inclusion gate) can then be established to exclude any debris above the threshold/discriminator but below the smallest lymphocytes. The CD45 versus SS plot (Fig. 5b) is then gated through Boolean gating on time and "not debris" with a gate drawn around the CD45+ cells. The CD45 versus SS plot is not only useful for pattern recognition, but is also useful for excluding any unlysed RBCs and other debris not removed by the debris exclusion gate. The CD15 versus SS plot (Fig. 5c) is gated on the CD45+ populations and includes a gate drawn around the CD15++ neutrophils excluding as well as possible the CD15 dim+ eosinophils visible to the left of the neutrophil/granulocyte population. The diagnostic FLAER/CD24 plot (Fig. 5d) is gated on the CD15++ neutrophils and a region is drawn to encompass the FLAER-negative/CD24-negative cells, which represent the PNH neutrophils. The CD64 versus SS plot (Fig. 5e) is also gated on the CD45+ cells and a region is drawn around the CD64++ monocytes. The FLAER versus CD14 dotplots (Fig. 5f) is gated on the CD64++ monocytes and a region is drawn to delineate the FLAER-negative/CD14-negative cells, which represent the PNH monocytes. The lymphocytes gated on the CD64-negative/low SS plot (5e) are not a suitable target population for the PNH clone quantification due to their long life span. However, they serve as internal controls for verification of antigen expression and compensation settings. Plotting FLAER versus CD24, CD14, CD15, and CD64 (5g–5k) verifies the instrument voltage and compensation settings as visible and clustered populations in the "correct" location. Plot 5g shows FLAER+/CD24+ B-cells verifying that both reagents were added, FLAER+/CD24-negative NK and T-cells and no dual-negative cells as this is a PNH negative sample.

Figure 6 shows the same process using the same Boolean gating strategy on a patient with a PNH clone showing in the diagnostic plots for both the neutrophils and monocytes. The lymphocytes (internal controls) also show a much smaller PNH clone in the dual-negative region and verifies that the instrument is optimized to show the negative cells on scale, thus verifying appropriated instrument voltage and compensation settings.

FLAER/CD157 Based Single Tube Assay for Neutrophils and Monocytes

ADP-ribosyl cyclase 2 (CD157) is a GPI-anchored cell surface enzyme encoded by the bone marrow stromal cell antigen-1 gene which plays a role in pre-B cell growth (19,20). Within the hematopoietic system, CD157 is highly expressed on both mature neutrophils and monocytes (21,22) leading to the possibility that CD157 could replace both CD24 and CD14, allowing the development of a single tube, high sensitivity 5C assay to identify and quantify both PNH neutrophils and PNH monocytes on cytometers with five or more PMTs (15–17,23). The ability to perform simultaneous evaluation of both PNH neutrophils and PNH monocytes is particularly attractive to laboratories equipped with 5-C instruments such as the FC500 due to the major cost and time savings involved over running two separate four-color assays for neutrophils and monocytes (15–17,23). The gating and analysis strategies are similar to the ones used for the above described single-tube six-color assay, except for the diagnostic FLAER/CD157 dotplots gated on CD15++ neutrophils and CD64++ monocytes (Fig. 7). Three control lymphocyte plots FLAER/CD157, FLAER, CD15, and FLAER CD64 are also shown to monitor instrument setup and compensation.

As detailed in Section "Distinguishing PNH Populations From Normal" and elsewhere (15–17) it is important to note that several CD157-negative, non-PNH cases have been observed in the authors' laboratories (unpublished data). For these rare cases, the inclusion of the

second GPI reagent (FLAER) as part of the built-in robustness of the assay prevents the mis-interpretation of the data as a PNH clone-containing sample; the loss of two GPI-linked structures is required to identify *bona fide* PNH phenotypes. Furthermore, in keeping with current state-of-the-art guidelines (9,10) the RBC lineage should also be analyzed on every sample tested for the presence of PNH WBCs. As these rare CD157-negative non-PNH samples only contain normal (Type I) RBCs, there is even less chance of mis-interpretation. An example of a CD157-negative case is shown in the (Case library, Case 14). The sample was stained with a seven-color combination of FLAER, CD157, CD24, CD14, CD15, CD64, and CD45. While the CD157 failed to stain normal neutrophils in this sample, both FLAER and CD24 stained in the expected manner. Similarly, while CD157 failed to stain normal monocytes in this sample, and FLAER and CD14 stained monocytes in the expected manner.

Type II Cells in Neutrophils and Monocytes

The clinical utility of determining both Type III and Type II PNH RBC clone sizes has been well established and reporting both individually as well as the total RBC PNH clone size is required whenever possible (9,13,14,16). Patients with bothType II (partial GPI deficiency) and Type III WBCs (complete GPI deficiency) may also be seen in the neutrophil and monocyte lineages. Patient samples with Type II neutrophils may also contain Type II monocytes but the levels of Type II WBCs and Type II RBCs rarely correlate. Just as it can be extremely difficult to delineate PNH Type III RBCs from PNH Type II, and PNH Type II from normal RBCs, it can also be difficult to delineate Type III, Type II and normal populations of neutrophils and monocytes and accurate gate setting can thus be problematic. Poor instrument setup/compensation settings can affect the location of the GPI deficient population and aging samples may show a wider distribution of the GPI-negative population "pushing" some cells into the Type II area . Furthermore, different reagent combinations (for example of CD157/FLAER and CD24/FLAER) derive different levels of Type II and Type III neutrophils on the same sample, as do CD157/FLAER and CD14/FLAER combinations for the monocyte lineage (Fig. 8). In some samples, Type II cells may be clearly delineated from Type III and from normal cells (see neutrophil plot bottom left in Fig. 8), but in others, the Type II cells may overlap either Type III cells when analysed with CD157 (Fig. 8a, neutrophils) or normal Type I cells (Fig. 8e–h) and gate setting can be quite problematic.

To date there are no clinical studies that support the utility of reporting PNH Type II neutrophils and/or PNH Type II monocytes. Moreover, there have been no proficiency testing programs, nor interlaboratory QC studies that have focused on the precision of PNH Type II neutrophil or PNH Type II monocyte results. For these reasons we think it is premature to recommend a standard procedure for reporting these populations. However, at the same time, we acknowledge that future research in correlating the presence of these populations with clinical outcome may be useful in determining if there is any clinical significance associated. It should be emphasized that these studies need to be performed under carefully controlled conditions to exclude artifacts due to age of the sample, poor viability, suboptimal instrument settings and specimen processing.

In conclusion, for clinical reporting purposes the authors strongly recommend to draw one PNH gate to include all cells with partial and/or complete GPI deficiency and report the total PNH clone size in neutrophils and monocytes.

WBC Assay QC—Use of Internal Controls

Optimal instrument setup and standardization in terms of PMT voltage optimization, daily monitoring and computer assisted spectral overlap compensation is a prerequisite for a validated flow cytometry assay. For this purpose, instrument platform based (BD CS&T module, BC AutoSetup Scheduler) or inter-platform (Euroflow, Harmonemia) approaches could be used (refer to Part 2). The use of a normal control sample is necessary to verify the assay performance for PNH RBC and WBC testing. However, the purpose of using lymphocytes as internal control populations verifies not only instrument performance in the test sample but also verifies that appropriate antibodies/reagents have been added to each individual patient sample and are performing in the expected manner. For protocols using FLAER in combination with CD24 for neutrophils and CD14 for monocytes as GPI specific markers, the FLAER versus CD24 dot plot, gated on lymphocytes can be used; the clear distinction of T lymphocytes/NK cells (FLAER+/CD24−), B lymphocytes (FLAER+/CD24+), and PNH lymphocytes (FLAER-/CD24-) is an excellent benchmark for all of the above parameters (Fig. 9). For protocols including FLAER in combination with CD157 for both neutrophils and monocytes as GPI specific markers, the FLAER versus CD157 dot plot, gated on lymphocytes can be less informative, since CD157 is not expressed on mature T and B lymphocytes. However, basophils are usually present in gated lymphocyte populations with this assay and they provide an internal control in situations where PNH clone sizes in neutrophils and monocytes approach 100%. Meanwhile, the delineation of PNH and non-PNH lymphocytes based on FLAER staining still provides reliable evidence of proper instrument setup (PMT voltage and compensation) and antibody specificity (Fig. 10). For protocols using the GPI specific markers CD157 with CD24 (for neutrophils) and CD14 (for monocytes) or three GPI specific markers (FLAER/CD24/CD157 for neutrophils and FLAER/CD14/CD157 for monocytes), the CD24 versus CD157 dot plot gated on all CD45+ leukocytes provides a means to assess assay quality; the clear distinction of B lymphocytes (CD24+/CD157−), T lymphocytes (CD24-/CD157, basophils (CD24−/CD157dim), monocytes (CD24−/

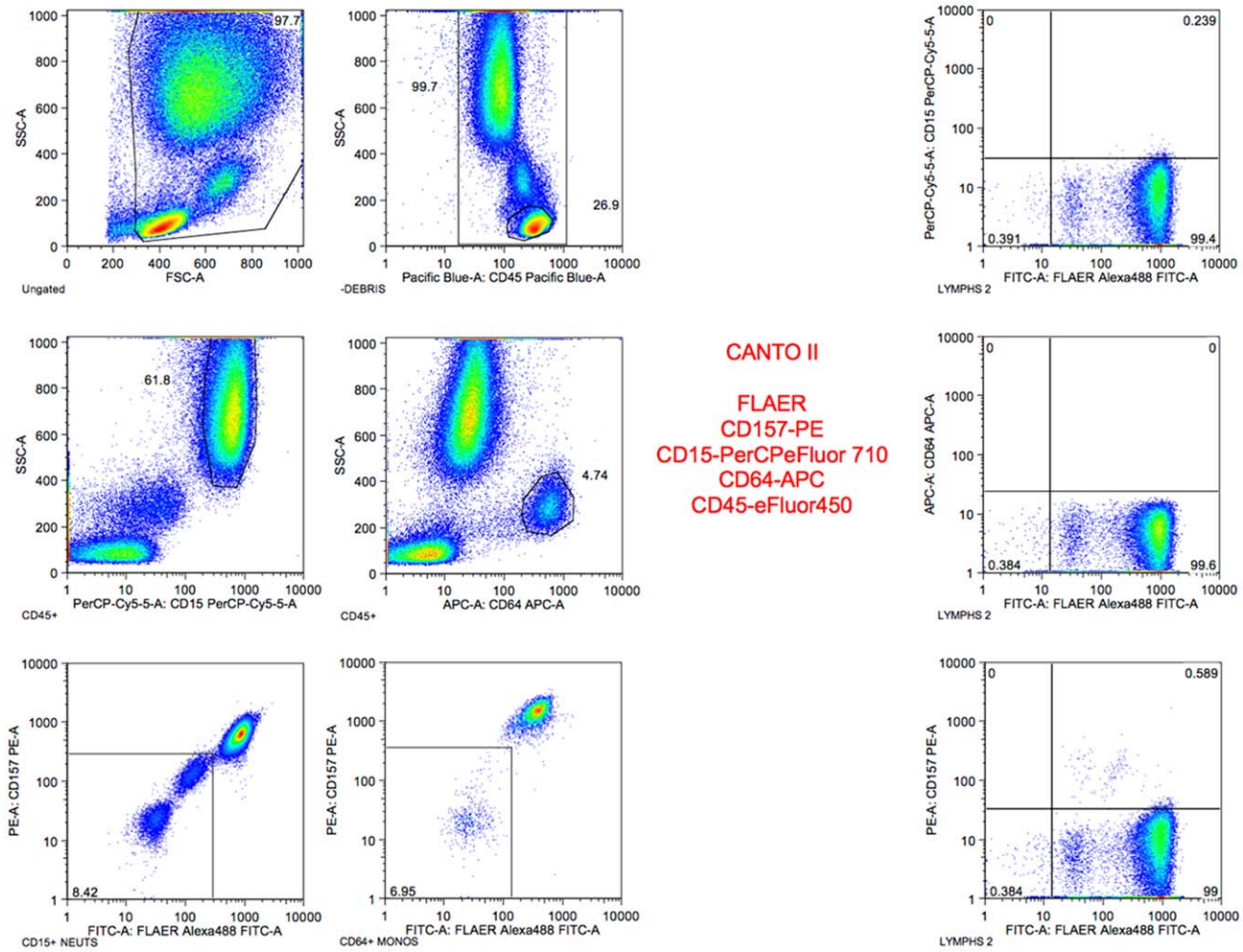


FIG. 7. PNH sample stained with five-color CD157-based assay. Sample contains Type III, and Type II PNH neutrophils (bottom left plot) but clearly defined Type II monocytes are not detectable (bottom middle). [Color figure can be viewed at wileyonlinelibrary.com]

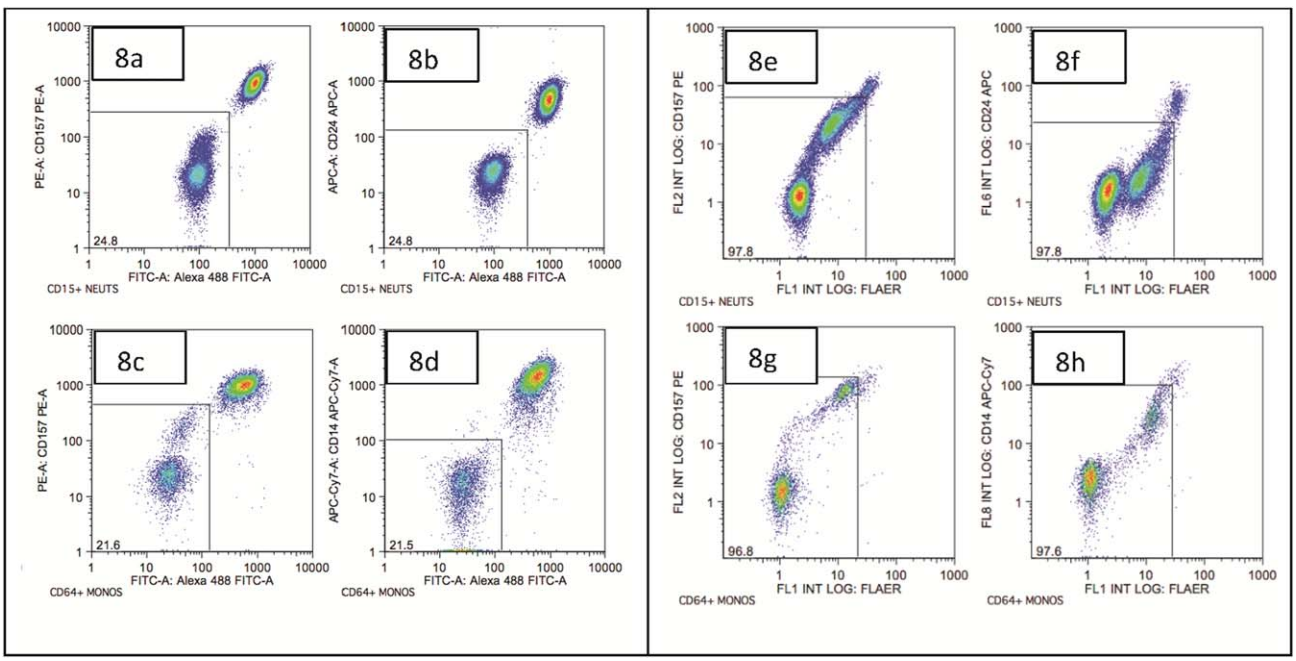


FIG. 8. PNH region setting for patients with Type II and Type III populations in the WBCs based on different combinations of GPI-specific reagents. Samples stained with seven-color assay as described in text. Patient A: CD15-gated neutrophils analyzed with CD157/FLAER (**a**) or with CD24/FLAER (**b**): CD64-gated monocytes from same tube analyzed with CD157/FLAER (**c**) or with CD14/FLAER (**d**). Patient B: CD15-gated neutrophils analyzed with CD157/FLAER (**e**) or with CD24/FLAER (**f**): CD64-gated monocytes from same tube analyzed with CD157/FLAER (**g**) or with CD14/FLAER (**h**). [Color figure can be viewed at wileyonlinelibrary.com]

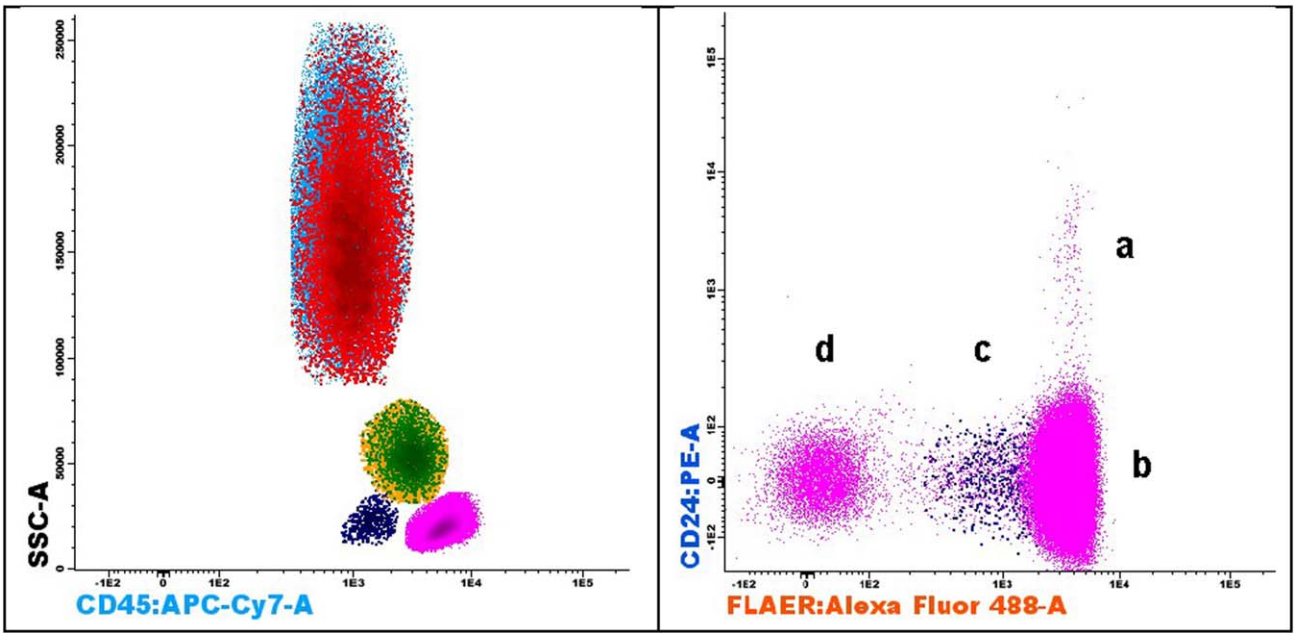


FIG. 9. Representative plots from a PNH assay using FLAER in combination with CD24 for neutrophils and CD14 for monocytes as GPI specific markers: B-lymphocytes (**a**), T-lymphocytes (**b**), NK cells and basophils (**c**), PNH lymphocytes (**d**). [Color figure can be viewed at wileyonlinelibrary.com]

CD157+), neutrophils (CD24+/CD157+), and eosinophils (CD24+/CD157 dim) can be delineated (Fig. 11).

Difference in PNH Clones Sizes Between Neutrophils and Monocytes

Over recent years more laboratories have reported patients with variability between PNH clone sizes in the neutrophils versus monocytes and such differences can be large. Traditionally the PNH neutrophil clone size has been used the "gold standard" and the main target population for assessing the clone size in the WBCs. However, as Figure 12 demonstrates, there is often significant variation between the two WBC populations which emphasizes the need for testing both populations for

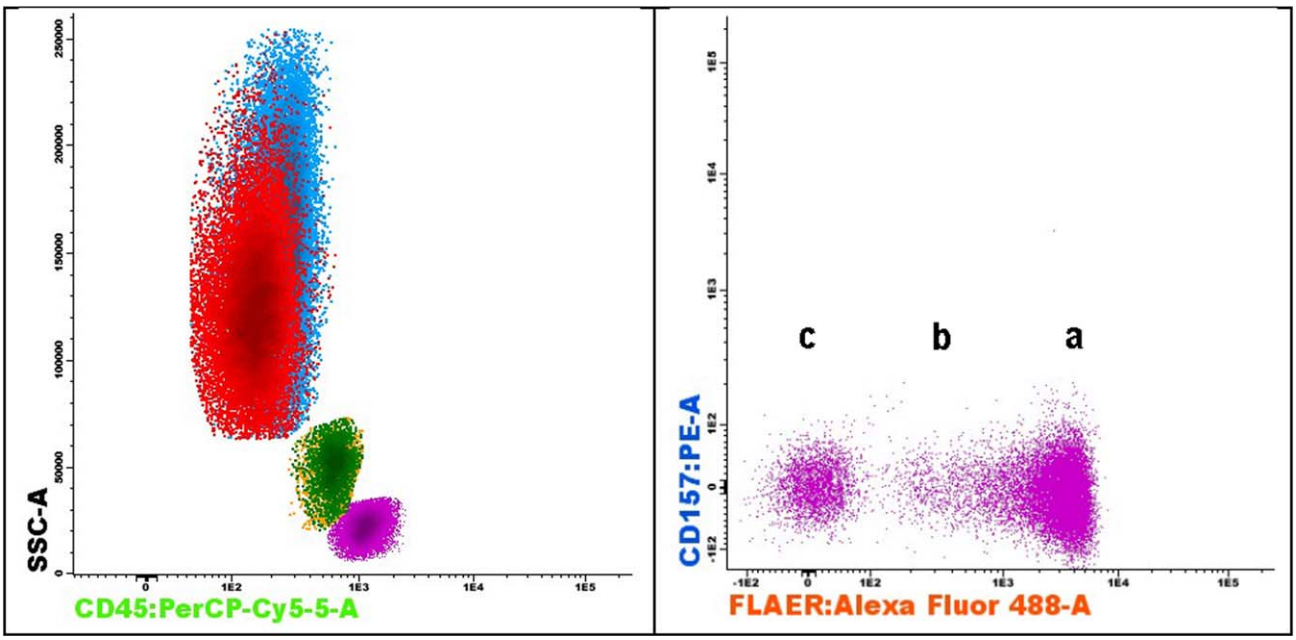


FIG. 10. Representative plots from a PNH assay using FLAER in combination with CD157 for both neutrophils and monocytes as GPI specific markers: lymphocytes (**a**), NK cells and basophils (**b**), PNH lymphocytes (**c**). [Color figure can be viewed at wileyonlinelibrary.com]

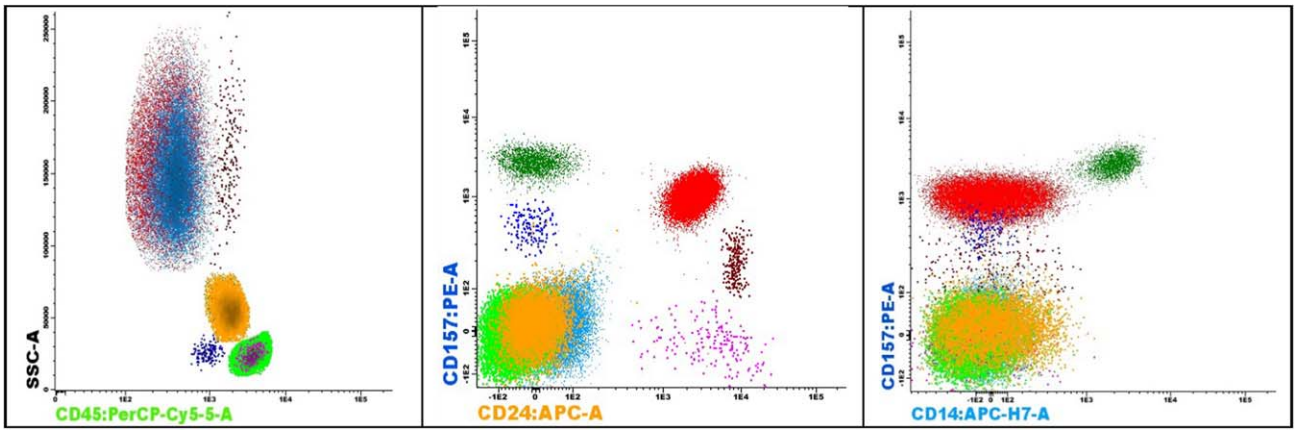


FIG. 11. Representative dot plots from a PNH assay using the combination of CD157 with CD24 (for neutrophils) and CD14 (for monocytes) as GPI specific markers: B-lymphocytes (purple), T-lymphocytes (chartreuse), basophils (blue), eosinophils (brown), non PNH neutrophils (red), non PNH monocytes (green), PNH neutrophils (cyan), PNH Mo (orange). [Color figure can be viewed at wileyonlinelibrary.com]

the presence of PNH phenotypes. Although there are some cases with higher clone sizes in the neutrophils, the majority of cases with differences have larger clone sizes in the monocytes. The reason for this is unclear at this time but possible factors may include infections, different lifespans, selective loss, and others. Reporting of both WBC populations from a single tube assay as well as the RBC clone size is recommended to avoid underestimation of the true PNH clone size in the WBCs, which might impact patient management.

Recommended Data Acquisition

1. The detection and quantification of PNH neutrophils and PNH monocytes is somewhat more challenging and complex compared to the RBC assay for a variety of reasons. While it is relatively easy to generate an LLOQ of 0.01% for the RBC assay, it is problematic to obtain the same sensitivity for neutrophil and monocyte assays. This problem is magnified in patients with neutropenia, where it may be difficult to acquire sufficient CD15++ neutrophils for analysis. In most cases there are sufficient neutrophils present and the acquisition of 50,000 CD15++ gated events is possible, which allows for a quantifiable assay with an LLOQ of 0.1% or better.
2. If there are no cells with PNH phenotype detected in 50,000 gated neutrophils, this can be reported as "no PNH cells detected" in the neutrophils. Based on Poisson statistics, there is a 99.3% probability that the true frequency of GPI-deficient cells is less than 0.01% at this level of acquired cells (9).
3. If there are fewer CD15++ neutrophils available for analysis due to neutropenia, the report should state the change in LLOQ and LOD and state that a minor population of PNH cells below the LOD cannot be ruled out (Table 1).

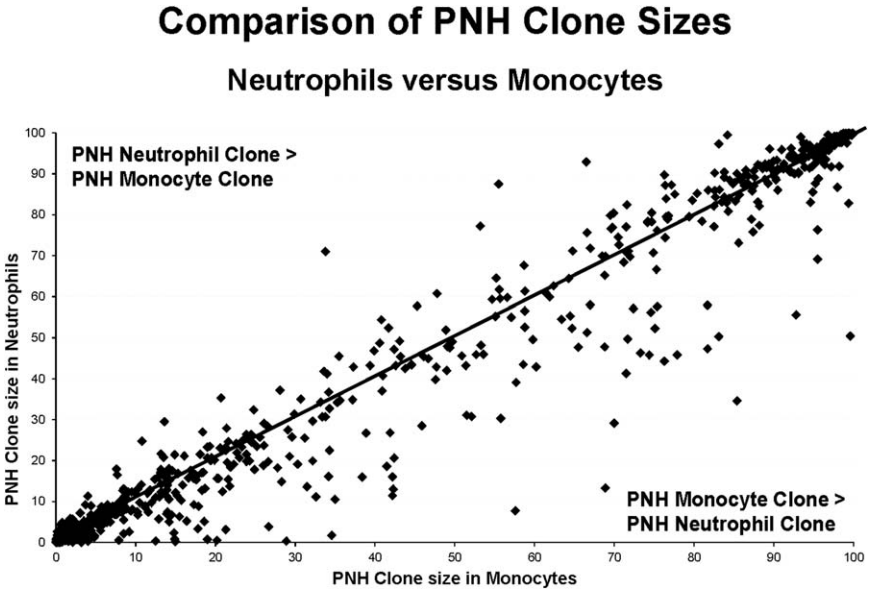


FIG. 12. Comparison of PNH neutrophil clone size and PNH monocyte clone size in 633 PNH-positive patients.

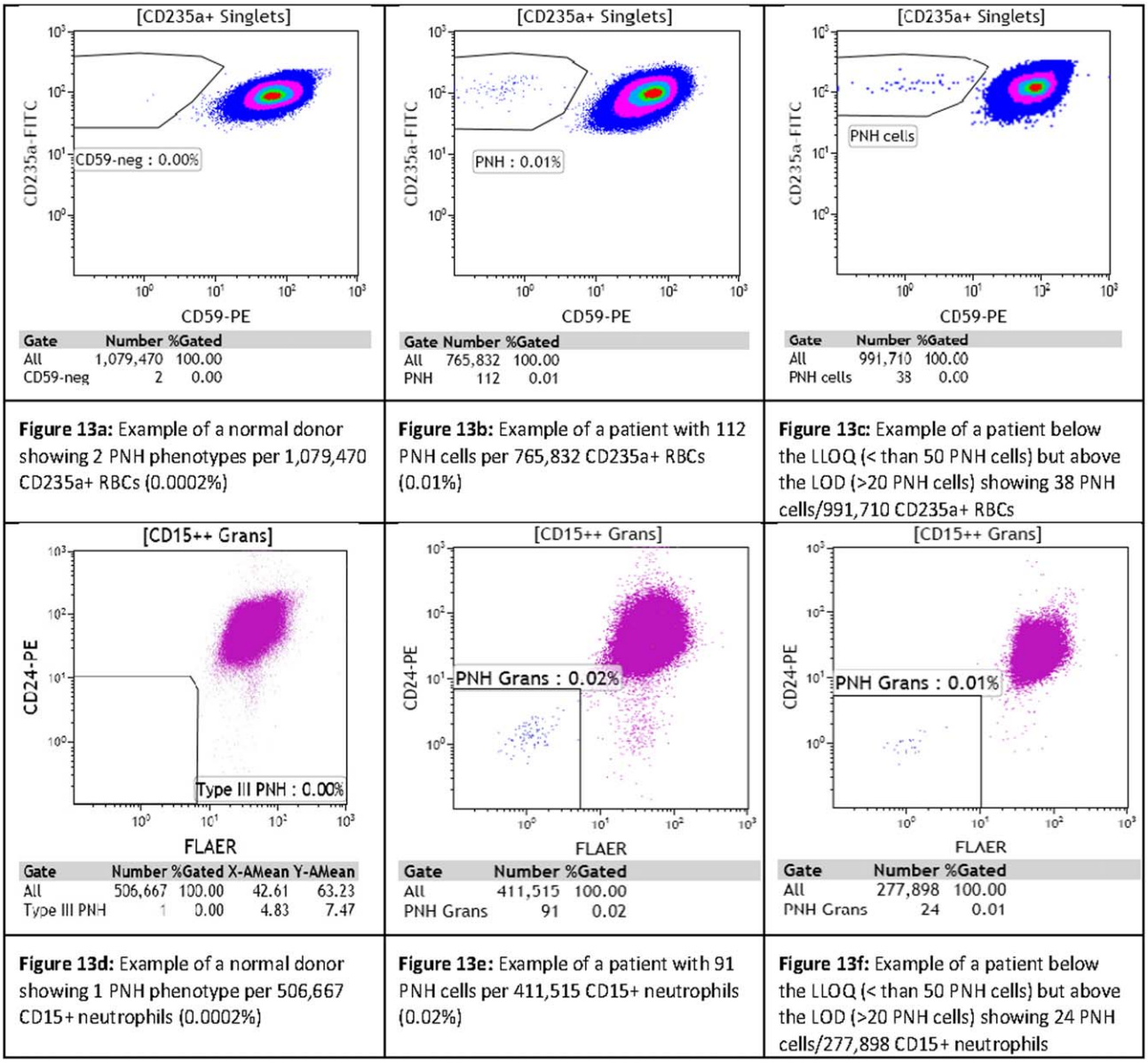


Figure 13a: Example of a normal donor showing 2 PNH phenotypes per 1,079,470 CD235a+ RBCs (0.0002%)

Figure 13b: Example of a patient with 112 PNH cells per 765,832 CD235a+ RBCs (0.01%)

Figure 13c: Example of a patient below the LLOQ (< than 50 PNH cells) but above the LOD (>20 PNH cells) showing 38 PNH cells/991,710 CD235a+ RBCs

Figure 13d: Example of a normal donor showing 1 PNH phenotype per 506,667 CD15+ neutrophils (0.0002%)

Figure 13e: Example of a patient with 91 PNH cells per 411,515 CD15+ neutrophils (0.02%)

Figure 13f: Example of a patient below the LLOQ (< than 50 PNH cells) but above the LOD (>20 PNH cells) showing 24 PNH cells/277,898 CD15+ neutrophils

FIG. 13. Incidence of GPI-deficient cells in normal donors versus minor populations of PNH cells. [Color figure can be viewed at wileyonlinelibrary.com]

4. If the laboratory chooses to provide a more sensitive assay, a greater volume of blood will need to be stained to allow for the acquisition of more cells (up to 500,000 cells for an LLOQ of 0.01%).
5. Using LLOQ: If more than 50 PNH cells are identified, a quantitative result (percentage) can be reported (Fig. 13e).
6. Using lower LOD: If at least 20 PNH phenotypes but less than 50 PNH phenotypes (as required for the LLOQ) are seen, it should be reported as "rare cells with PNH phenotype detected below the limit of quantification" (Fig. 13f).
7. The recommended LLOQ for monocytes is approximately 0.5% (based on acquisition of 10,000 monocytes) but for most patients the number of monocytes acquired may be quite variable. In most cases, in which minor clones are detected in the RBCs and neutrophils, insufficient monocytes are available for the detection of a minor clone. However, there are some instances in which more monocytes than neutrophils are available for analysis and they may become the primary target population for PNH clone detection and quantification.

It has been the authors' experience, that when small numbers of PNH phenotypes are detected, they are typically present in at least the RBC and neutrophils lineages. Although these small PNH populations may not be quantifiable, these patients should be

monitored and retested in accordance with international guidelines (i.e., Killick et al., Borowitz et al.).

MINOR PNH CLONES

Regardless of lineage, when <1% of the target population is GPI-deficient, PNH clone sizes have been historically referred to as "minor" (14,16). Such small populations of GPI-deficient cells are often encountered in patients with AA and some subsets of MDS (8–11). Clinically, these patients do not usually show symptoms of hemolysis but the presence of minor clones in AA is associated with better response to immunosuppressive therapy (24). They also should be monitored at specific intervals for possible clone size expansion to identify any patients who progress to clinical PNH (12). From a technical perspective, the same PNH RBC and WBC reagent panels can be used to screen all patient samples. However, for those containing only small numbers of PNH phenotypes, the number of events acquired may need to be increased depending on the sensitivity of the assay as established by the laboratory. The PNH assay is generally considered a quasi-quantitative PNH assay (Part 4) and the sensitivity of such assays is the percentage based on the LLOQ. However, as noted above, in some instances in which the number of PNH phenotypes detected is below the LLOQ but above the lower limit of LOD the assay does not behave as a quasi-quantitative assay and results can only be reported qualitatively, i.e., as positive below the level of quantitation. The characteristics of quasi-quantitative assays and the different relevant parameters are described in more detail in the validation section of these guidelines (Oldaker et al. op. cit.). Figure 13 shows an example of a RBC assay from a normal patient showing a background level (LOB) of 2 PNH phenotypes/1,079,470 gated events (Fig. 13a), a patient with the presence of rare (0.01%) but quantifiable PNH cells with >50 PNH cells (Fig. 13b) and a patient with rare PNH cells (>20 PNH cells but <50 PNH cells) which is detectable but not quantifiable (Fig. 13c). This is also illustrated for neutrophils, which shows an example of a normal sample showing a background level (LOB) of 1 PNH phenotype/506,667 gated events (Fig. 13d), a patient with the presence of rare (0.02%) but quantifiable PNH cells with >50 PNH cells (Fig. 13e) and a patient with rare PNH cells (>20 PNH cells but <50 PNH cells) which is detectable but not quantifiable (Fig. 13f).

Table 1 provides guidance regarding the number of acquired events necessary to determine the LLOQ. The LLOQ should be included in each report.

REPORTING

The following components are recommended for a PNH report:

1. Report if a PNH clone present or absent. It is important to be clear and to avoid potentially misleading ambiguous terminology. A report stating that a CD59 test is negative may imply to some providers that the target population is negative for the GPI marker CD59 (thus indicating a PNH clone) or that no CD59 absence is seen (thus indicating the absence of a PNH clone).
2. Report the PNH clone size in the RBCs (total PNH clone size as well as the percentages for Type II and Type III PNH populations). There is a clinical significance associated for Type II and Type III RBCs. Type I RBCs are normal RBCs with bright CD59 expression and a lifespan of approximately 120 days. Type III PNH RBCs have complete CD59 deficiency, which results in no protection from complement-mediated lysis and a shortened lifespan of 10–15 days. Type II PNH RBCs have partial CD59 deficiency resulting in partial protection from complement mediated lysis. Just as the expression of CD59 on Type II RBCs varies considerably from patient to patient, the lifespan of Type II cells reflects this being intermediate between Type I normal RBCs and Type III PNH RBCs. Since the clinical significance of Type II PNH RBCs and Type III PNH RBCs is well established, it is recommended to report them separately and combined as the total PNH RBC clone.
3. Report the PNH clone size in both lineages for the WBCs (neutrophils and monocytes). The PNH monocyte clone is often larger than the neutrophils PNH clone and reporting only the PNH neutrophil clone may underestimate the PNH clone size in the WBCs. Neutrophils and monocytes may also show the presence of Type II populations but the clinical and biological significance of these populations has not been established at this time. It is therefore recommended to report only the total PNH clone size in the neutrophils and monocytes.
4. Interpretive terminology of reporting PNH clones based on CSLI H52-A2 (14):
   a. PNH population >1%: "PNH clone."
   b. PNH population 0.1% to 1%: "minor population of PNH cells" or "minor PNH clone."
   c. PNH population <0.1%: "rare cells with GPI deficiency" or "rare cells with PNH phenotype."
5. List all gating and diagnostic markers used for the PNH assay.
6. State the LLOQ for the neutrophil/granulocyte assay and the RBC assay on the report, stating the recommended LLOQ of 0.05% or better for RBCs (100,000 gated cells) and 0.1% or better for neutrophils (50,000 gated cells). It is important to include this information to the provider as an LLOQ of 1% means that the possibility of a minor clone (less than 1%) cannot be excluded based on this LLOQ.
7. Histograms or dotplots: These are optional as many laboratory information systems are not able to include images. However; the dotplots may provide powerful visual supportive evidence of the PNH clone and also provide evidence of the quality of the assay.
8. Retesting recommendations: Appendix A (uploaded in the online version) shows an example of a generic reporting template that meets current recommendations and requirements. Also included are current recommendations based in International Guidelines for re-testing frequencies in PNH and related diseases.

SAMPLE REPORTS

The following four cases represent the most frequently encountered findings when testing for PNH. The report contains all required components as listed above. The comment section may be used to describe some of the more unusual findings if encountered.

SAMPLE REPORT 1 FOR PATIENT WITH NO PNH CLONE

RESULTS: NO PHENOTYPIC EVIDENCE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

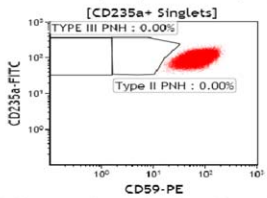
Comment: Flow cytometric analysis does not show any evidence of a PNH clone based upon analysis of a variety of GPI-linked antibodies on red blood cells, monocytes and neutrophils. These findings do not support a diagnosis of PNH. Clinical correlation is recommended.

Reference: 1. Borowitz et al: Guidelines for the Diagnosis and Monitoring of PNH and Related Disorders, Clin Cytometry 2010, 211-230
2. Sutherland et al: Practical guidelines for the high-sensitivity detection and monitoring of PNH clones by flow cytometry. Cytometry B Clin Cytom 2012; 82:195-208.
3. http://www.pnhsource.com/physicians

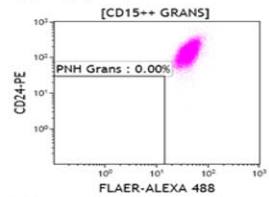
Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24 and FLAER.

Cell population	Result	LLOQ
CD235a+ RBC	No CD59 negative RBCs identified	0.01%
CD64+ Monocytes	No FLAER/CD14-negative cells	0.1% *
CD15+ Neutrophils	No FLAER/CD24-negative cells	0.01% *

The Lower Limit of Quantitation (LLOQ)* of WBC assay may be decreased in severely pan-cytopenic patients.



Sample histogram of a typical patient with no evidence of PNH in RBC's



Sample histogram of a typical patient with no evidence of PNH in Neutrophils

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

[Color figure can be viewed at wileyonlinelibrary.com]

SAMPLE REPORT 2 FOR PATIENT WITH PNH CLONE

RESULTS: PNH CLONE IDENTIFIED IN WBC AND RBC

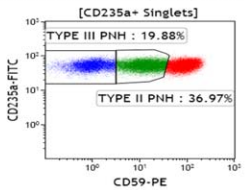
Comment: Flow cytometric analysis shows a PNH clone within the neutrophils (79.5%), monocytes (88.6%) and RBC (56.9%). These findings are consistent with a diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). Any potential difference in clone size between the white blood cells and the red blood cells may be due to hemolysis and/or recent transfusion.

Reference: 1. Borowitz et al: Guidelines for the Diagnosis and Monitoring of PNH and Related Disorders, Clin Cytometry 2010, 211-230
2. Sutherland et al: Practical guidelines for the high-sensitivity detection and monitoring of PNH clones by flow cytometry. Cytometry B Clin Cytom 2012; 82:195-208.
3. http://www.pnhsource.com/physicians

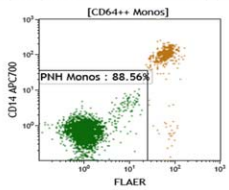
Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24, as well as fluorescent Aerolysin (FLAER).

Cell Type	Deficiency	Result	LLOQ
RBC	Type II (partial CD59 deficiency)	37.0%	NA
	Type III (complete CD59 deficiency)	19.9%	NA
	Total RBC PNH Clone size (Type II and Type III)	56.9%	0.01%
WBC- Monocytes	FLAER/CD14 Deficiency	88.6%	0.1% *
WBC- Neutrophils	FLAER/CD24 Deficiency	79.5%	0.01% *

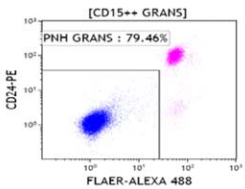
The Lower Limit of Quantitation (LLOQ)* of WBC assay may be decreased in severely pan-cytopenic patients.



Type III and Type II PNH RBC clones



PNH clone (green) in Neutrophils



PNH clone (blue) in Neutrophils

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

[Color figure can be viewed at wileyonlinelibrary.com]

SAMPLE REPORT 3 FOR PATIENT WITH FOLLOW-UP OF PNH CLONE

RESULTS: **1. PNH CLONE IDENTIFIED IN WBC**
2. MINOR POPULATION OF PNH CELLS IDENTIFIED IN RBC

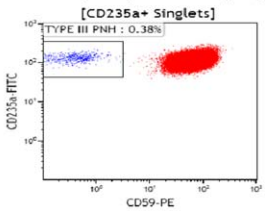
Comment: Flow cytometric analysis shows a PNH clone within the neutrophils (1.4%), monocytes (1.3%) and a minor PNH clone in the RBC (0.4%). The clinical significance of the small populations with GPI deficiency is uncertain at this time; recommend repeat testing in 3-6 months. Clinical correlation is recommended.

Reference: 1. Borowitz et al: Guidelines for the Diagnosis and Monitoring of PNH and Related Disorders, Clin Cytometry 2010, 211-230
2. Sutherland et al: Practical guidelines for the high-sensitivity detection and monitoring of PNH clones by flow cytometry. Cytometry B Clin Cytom 2012; 82:195-208.
3. http://www.pnhsource.com/physicians

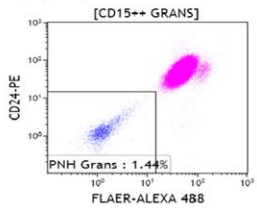
Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24, as well as fluorescent Aerolysin (FLAER).

Cell Type	Deficiency	Current	Previous	Previous	Previous	LLOQ
RBC	CD59 Deficiency	0.4%	0.14%	0.07%	0.06%	0.01%
Monocytes	FLAER/CD14 Deficiency	1.3%	0.51%	0.23%	0.20%	0.1% *
Neutrophils	FLAER/CD24 Deficiency	1.4%	0.58%	0.24%	0.18%	0.01% *

The Lower Limit of Quantitation (LLOQ)* of WBC assay may be decreased in severely pan-cytopenic patients.



Minor Type III (blue) PNH clone in RBC's



PNH clone (blue) in Neutrophils

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

[Color figure can be viewed at wileyonlinelibrary.com]

SAMPLE REPORT 4 FOR PATIENT WITH RARE PNH CELLS IN NEUTROPHILS AND RED BLOOD CELLS

RESULTS: **1. RARE CELLS WITH PNH PHENOTYPE DETECTED IN RBC**
2. RARE CELLS WITH PNH PHENOTYPE IDENTIFIED IN WBC

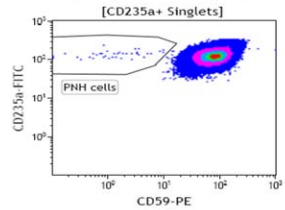
Comment: Flow cytometric analysis shows rare cells with PNH phenotype in the RBC and neutrophils. The clinical significance of these minute populations with GPI deficiency is uncertain at this time; however, an evolving PNH clone cannot be entirely excluded. Consider repeat testing in 6-12 months. Clinical correlation with other laboratory data and follow-up is recommended.

Reference: 1. Borowitz et al: Guidelines for the Diagnosis and Monitoring of PNH and Related Disorders, Clin Cytometry 2010, 211-230
2. Sutherland et al: Practical guidelines for the high-sensitivity detection and monitoring of PNH clones by flow cytometry. Cytometry B Clin Cytom 2012; 82:195-208.
3. http://www.pnhsource.com/physicians

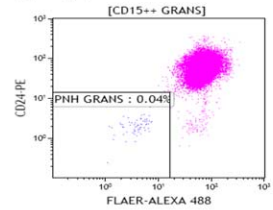
Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24 and FLAER.

Cell population	Result	LLOQ
CD235a+ RBC	Rare cells with PNH phenotype detected (below LLOQ)	0.01%
CD64+ Monocytes	No FLAER/CD14-negative cells detected	0.1% *
CD15+ Neutrophils	0.04% PNH cells identified	0.01% *

The Lower Limit of Quantitation (LLOQ)* of WBC assay may be decreased in severely pan-cytopenic patients.



38 PNH cells/991,710 total RBC



68 PNH cells/177,638 total Neutrophils

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

[Color figure can be viewed at wileyonlinelibrary.com]

CASE LIBRARY

The case studies (uploaded in the online version) are derived from several patients screened for PNH. Some of these cases are easy to interpret (see case study 1 and 2), others present a more challenging interpretation and reflect the heterogeneity of cases screened for the presence of a PNH clone. The purpose is to provide the reader of this document with a variety of situations and interpretational suggestions. Most reference laboratories do not receive clinical data with the sample so this is strictly an interpretation based on technical data.

CONCLUSION

Highly sensitive and specific PNH testing of all three lineages (RBC, neutrophils, and monocytes) has become the standard of care for patients with suspected PNH. This is a rare disease and therefore often overlooked as a diagnostic possibility. It is important for the ordering physician to test the high-risk patients for PNH (8,9) and also to receive informational reports as an important part for best patient management. The laboratories are challenged with the validation of multiple steps, including instrument optimization, selection of best antibody clones/conjugates, panel design and targeted acquisition and interpretation of data. Developing competency in PNH testing and reporting is critical for laboratories and is directly related to awareness of best practices, following guidelines which are developed by experts based on extensive evaluation of various approaches. Standardized reporting based on currently available guidelines is important to communicate to the physician the size of the PNH clone, which aids him/her in the decision-making for optimal treatment of the patient.

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